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Overexpression of cFLIPs Inhibits Oxaliplatin-Mediated Apoptosis Through Enhanced XIAP Stability and Akt Activation in Human Renal Cancer Cells

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ABSTRACT

cFLIP inhibits caspase 8 recruitment and processing at the death-inducing signaling complex (DISC), which is known to inhibits apoptosis mediated by death receptors such as Fas and death receptor 5 (DR5) as well as apoptosis mediated by anticancer therapeutic drugs. We observed that oxaliplatin induced apoptosis, the activation of DEVDase activity, DNA fragmentation, and cleavage of PLC-γ1 and degradation of XIAP protein in dose-dependent manners, which was prevented by pretreatment with z-VAD or NAC, suggesting that oxaliplatin-induced apoptosis was mediated by caspase- or reactive oxygen species (ROS)-dependent pathways. Furthermore, ectopic expression of cFLIPs potently attenuated oxaliplatin-induced apoptosis, whereas $cFLIP_L$ had less effect. Interestingly, we found that the protein level of XIAP was sustained in oxaliplatin-treated cFLIPs overexpressing cell, which was caused by the increased XIAP protein stability and that the phospho-Akt level was high compared to vector-transfected cell. The increased XIAP protein stability was lessened by PI3K inhibitor LY294002 treatment in cFLIPs overexpressing cells. Thus, our findings imply that the anti-apoptotic functions of cFLIPs may be attributed to inhibit oxaliplatin-induced apoptosis through the sustained XIAP protein level and Akt activation. J. Cell. Biochem. 105: 971–979, 2008.

KEY WORDS: cFLIPs; OXALIPLATIN; APOPTOSIS; XIAP; Akt

cFLIP is catalytically inactive homolog of caspase-8, which inhibits caspase 8 recruitment and processing at the DISC [Krueger et al., 2001]. cFLIP encodes two splicing variants, long form of cFLIP ($cFLIP_L$) and short form of cFLIP (cFLIPs). While cFLIPs only contains two N-terminal death effector domains (DEDs), $cFLIP_L$ consists of the N-terminal DEDs and a C-terminal caspase-like domain that does not possess enzymatic activity [Goltsev et al., 1997]. cFLIPs directly inhibits caspase 8 activation at the DISC, whereas $cFLIP_L$ is first processed to a truncated p43 form by caspase 8 that inhibits the complete processing of caspase 8 to its active subunits. Overexpression of both long and short isoform of cFLIP has been shown to protect against apoptosis mediated by death receptor including FasL and TRAIL in several cancer cells in vitro [Irmler et al., 1997; Shu et al., 1997; Medema, 1999].

Recently, it has been demonstrated that the cFLIP variants perform differential roles in regulating apoptosis [Krueger et al., 2001; Jin et al., 2004]. For instance, whereas cFLIPs has been shown to inhibit TRAIL-induced DISC formation and apoptosis [Burns and El-Deiry, 2001; Bin et al., 2002], the role of $cFLIP_L$ at the DISC is still a matter of controversy. Some reports describe it as an anti-apoptotic molecule that inhibits Fas-induced caspase-8 recruitment and activation, whereas others describe it as a proapoptotic molecule, facilitating the activation of procaspase-8 at the DISC [Scaffidi et al., 1999; Kataoka et al., 2000; Chang et al., 2002]. In addition, cFLIP proteins were invoked to play a prominent role in NF-κB and the mitogen-activated protein kinase ERK signaling pathways and to control life or death decisions [Hu et al., 2000; Kataoka et al., 2000; Golks et al., 2006]. Recently, it has been

Abbreviations used: ROS, reactive oxygen species; TRAIL, tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand; cIAP, cellular inhibitor of apoptosis protein; HSC70, 70-kDa heat shock cognate protein; cFLIP, cellular FLICE inhibitory protein; PLC-γ1, phospholipase C-γ1; XIAP, X-linked inhibitor of apoptosis protein; z-VAD-fmk, Benzyloxy carbony-Val-Ala-Asp-fluoromethyl ketone; NAC, *N*-acetylcysteine; H_2DCFDA, 2′,7′-dichlorodihydro-fluorescein diacetate.

Shin Kim and Tae-Jin Lee contributed equally this work.

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reported that overexpression of $cFLIP_L$ potently inhibits chemotherapeutic drugs-induced cell death in solid tumor-derived cancer cell lines [Matta et al., 2002; Longley et al., 2006]. However, the molecular events and the exact underlying mechanisms responsible for cFLIPs-mediated protection against various anti-cancer drugs are poorly understood. Here, we show that overexpression of cFLIPs attenuates oxaliplatin-induced apoptosis in human renal Caki cells through the increased XIAP protein stability and the activation of Akt. These findings might provide a new mechanism of cFLIPs-mediated protection against anti-cancer drugs and shed light on the regulation of life/death decisions made in renal cancer cells.

MATERIALS AND METHODS

CELLS AND MATERIALS

Human renal carcinoma Caki cells were obtained from ATCC (Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. The oxaliplatin (OXA) was directly added to cell cultures at the indicated concentrations while untreated cells contained the solvent alone. Anti-PLC-γ1, anti-procaspase-3, anti-Bcl-2, and anti-Mcl-1antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody against XIAP proteins was obtained from R&D systems (Minneapolis, MN), and benzyloxycarbony-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) from Biomol (Plymouth Meeting, PA). Oxaliplatin was purchased from Calbiochem (San Diego, CA).

cFLIP CONSTRUCTS AND STABLE CELL LINES

The human $cFLIP_L$ and cFLIPs cDNA fragments were digested from pCA-FLAG-$hFLIP_L$ and pCA-FLAG-hFLIPs. pCA-FLAG-$hFLIP_L$ and pCA-FLAG-hFLIPs were kindly provided by Dr. Park SI (Korea Centers for Disease Control and Prevention, Seoul, Korea). pCA-FLAG-$hFLIP_L$ and cFLIPs cDNA fragment were digested with *Kpn*I and *Xho*I and subcloned into the pcDNA 3.1(+) vector (Invitrogen, Carlsbad, CA), termed pcDNA 3.1(+)-$cFLIP_L$ and pcDNA 3.1(+)-cFLIPs. The Caki cells were transfected in a stable manner with the pcDNA 3.1(+)-$cFLIP_L$ and pcDNA 3.1(+)-cFLIPs plasmid, or control plasmid pcDNA 3.1(+) Neo vector using LipofectAMINE as prescribed by the manufacturer (Invitrogen). After 48 h of incubation, transfected cells were selected in primary cell culture medium containing 700 μg/ml G418 (Invitrogen). After 2 or 3 weeks, to rule out the possibility of clonal differences between the generated stable cell lines, the pooled Caki/pcDNA 3.1, Caki/cFLIPs, and Caki/$cFLIP_L$ clones were tested for cFLIPs and $cFLIP_L$ expression by immunoblotting and were used in this study.

WESTERN BLOTTING

Cellular lysates were prepared by suspending 1×10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl2, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride, and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

FLOW CYTOMETRY ANALYSIS

Approximately 1×10^6 Caki cells were suspended in 100 μl PBS, and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4°C for 1 h, washed with PBS, and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

Asp-Glu-Val-Asp-ase (DEVDase) ACTIVITY ASSAY

To evaluate DEVDase activity, cell lysates were prepared after their respective treatment with oxaliplatin. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspases substrate [Asp-Glu-Val-Asp-chromophore-*p*-nitroanilide (DVAD-pNA)] at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

CELL DEATH ASSESSMENT BY DNA FRAGMENTATION ASSAYS

The cell death detection ELISA plus kit (Boerhringer Mannheim, Indianapolis, IN) was used for assessing apoptotic activity by detecting fragmented DNA within the nucleus in oxaliplatin-treated cells. Briefly, each culture plate was centrifuged for 10 min at 200*g*, the supernatant was removed, and the pellet was lysed for 30 min. After centrifuging the plate again at 200*g* for 10 min, the collected supernatant containing cytoplasmic histone-associated DNA fragments was incubated with an immobilized anti-histone antibody, and the reaction products were determined by spectrophotometry. Finally, absorbance at 405 and 490 nm (reference wavelength), upon incubating with a peroxidase substrate for 5 min, was determined with a microplate reader. Signals in the wells containing the substrate only were subtracted as background.

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS)

The intracellular accumulation of ROS was determined using the fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA was commonly used to measure H_2O_2 [Sauer et al., 2003; Kim et al., 2005]. H_2O_2 in the cells cause oxidation of DCFH-DA, yielding the fluorescent product 2′,7′-dichlorofluorescein (DCF). Briefly, Caki cells were incubated with oxaliplatin for 24 h, collected by centrifugation and resuspended in DMEM medium without red phenol, and loaded with 5 μM H_2DCFDA 1 h before harvesting. The fluorescence was measured at the desired time intervals by flow cytometry. The ROS generation was assessed by the dichlorofluorescein fluorescence intensity (FL-1, 530 nm) from 10,000 cells with a FACSCalibur flow cytometer (Becton Dickinson). Control cells were subjected to the same manipulation, except for treatment with the oxaliplatin.

RNA ISOLATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Total cellular RNA was extracted by using the TRI reagent. Single-strand cDNA was synthesized from 2 μg of total RNA using M-MLV (Moloney–Murine leukemia virus) reverse transcriptase. The cDNA for XIAP was PCR amplified using the following specific primers: XIAP (sense) 5′-CTTGAGGAGTGTCTGGTAA-3′ and (antisense) 5-GTGACTAGATGTCCACAA GG-3. PCR amplification was carried out as follows: 1× (94°C, 3 min); 30× (94°C, 45 s; 59°C, 45 s; and 72°C, 1 min); and 1× (72°C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

RESULTS

OXALIPLATIN INDUCES APOPTOSIS IN Caki CELLS

Human renal carcinoma Caki cells were treated with various concentrations of the oxaliplatin (50–125 μM). Treatment with oxaliplatin exhibited progressive morphological changes of typical apoptosis, including cell shrinkage, rounding, and detachment of the cells from the plate, as observed with light microscopy (Fig. 1A). We determined apoptosis in Caki cells using flow cytometric analysis to detect sub-G1 phase cells. As shown in Figure 1B, treatment of Caki cells with oxaliplatin resulted in a markedly increased accumulation of sub-G1 phase cells in a dose-dependent manner. We next analyzed whether treatment with oxaliplatin caused the activation of caspases, a key executioner of apoptosis. Exposure of Caki cells to oxaliplatin strongly stimulated DEVDase activity (Fig. 1C). Furthermore, treatment with oxaliplatin for 24 h increased cytoplasmic histone-associated DNA fragments as determined with the DNA fragmentation detection test (Fig. 1D). As shown in Fig. 1E, treatment with 50–125 μM oxaliplatin for 24 h led to a reduction of the protein levels of XIAP and 32-kDa caspase-3 precursor together with a concomitant cleavage of phospholipase C-γ1 (PLC-γ1), a substrate protein of caspases. However, protein levels of apoptotic regulators including cIAP1, cIAP2 and Mcl-1 were not affected by treatment with oxaliplatin.

OXALIPLATIN-INDUCED APOPTOSIS IS MEDIATED BY CASPASE-DEPENDENT PATHWAY AND GENERATION OF REACTIVE OXYGEN SPECIES (ROS)

To address the significance of caspase activation in oxaliplatin-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Aspfluoromethyl ketone). As shown in Figure 2A, oxaliplatin-induced apoptotic population was significantly inhibited by pretreatment with z-VAD-fmk. Not only oxaliplatin induced increase of DEVDase activity (Fig. 2B), but also it induced the degradation of pro-caspase-3 and produced 60 kDa cleavage product of PLC-γ1 (Fig. 2C). These results suggest that oxaliplatin-induced apoptosis is mediated by caspase-dependent pathway.

Numerous investigations have documented that oxidative stress-mediated cellular changes are frequently induced in cells exposed to cytotoxic drugs, UV or γ-irradiation [Sheng-Tanner et al., 1998; Wen et al., 2002]. We here examined whether oxaliplatin affects the cellular levels of peroxide by measuring the changes in the fluorescence using H_2DCFDA. As shown in Figure 2D, treatment with oxaliplatin markedly increased the H_2DCFDA-derived fluorescence. This oxaliplatin-mediated increase in fluorescence was markedly inhibited by pretreatment with anti-oxidant *N*-acetylcysteine (NAC). Then, we next asked whether ROS generation induced by oxaliplatin is directly associated with the induction of apoptosis. As shown in Figure 2E,F, pretreatment with NAC prevent oxaliplatin-induced increase of sub-G1 population and increase of DEVDase activity. These data clearly indicate that ROS generation is critical for oxaliplatin-induced apoptosis.

cFLIPs OVEREXPRESSION ATTENUATES OXALIPLATIN-INDUCED APOPTOSIS

Overexpression of cFLIP is known to inhibit chemotherapy-induced apoptosis [Matta et al., 2002; Longley et al., 2006]. To determine the effect of treatment with oxaliplatin on the viability of cFLIP-overexpressing cells, we employed Caki cells engineered for overexpression of cFLIPs (Caki/ cFLIPs), $cFLIP_L$ (Caki/$cFLIP_L$), and vector-transfected control cells (Caki/Vector) (Fig. 3A). Stable cFLIPs- and $cFLIP_L$-overexpressing cell lines were treated with various concentrations of oxaliplatin or TRAIL, and examined cytotoxicity using FACS analysis. As shown in Figure 3B, oxaliplatin treatment in Caki/Vector cells resulted in a markedly increased accumulation of sub-G1 phase in a dose-dependent manner. Overexpression of cFLIPs significantly attenuated oxaliplatin-induced accumulation of sub-G1 phase, whereas overexpression of $cFLIP_L$ slightly protected oxaliplatin-induced apoptosis (Fig. 3B). As shown in Figure 3C, the inhibition of TRAIL-mediated apoptosis was stronger in cFLIPs overexpressing cells than in $cFLIP_L$ cells. These results indicate that cFLIPs is the critical splice form in mediating chemoresistance to oxaliplatin in Caki cells. Next, we further studied on the protection mechanism of cFLIPs in oxaliplatin-treated Caki cells. We measured DEVDase activity in Caki/Vector and Caki/cFLIPs cells exposed to 50–125 μM oxaliplatin for 24 h. Figure 4A showed that the DEVDase activities were lower in cFLIPs overexpressing cells than control cells. Western blotting assay showed proteolytic cleavage of PLC-γ1 and decrease of procaspase-3 in Caki/Vector cells after 24 h treatment with oxaliplatin. In contrast, the level of 60 kDa PLC-γ1 cleavage product was evidently reduced and the level of procaspase-3 was sustained in Caki/cFLIPs cells (Fig. 4B). Furthermore, as shown in Figure 4B, while oxaliplatin treatment in Caki/Vector cells resulted in a markedly decreased XIAP protein levels, overexpression of cFLIPs reduced oxaliplatin-induced XIAP down-regulation. We also examined whether cFLIPs could modulate the expression of Mcl-1 and Bcl-2, which ultimately determine the cellular response to apoptotic stimuli. Treatment of Caki/Vector and Caki/cFLIPs cells with oxaliplatin at concentrations that are sufficient to induce apoptosis failed to significantly alter the expression of the Bcl-2 and Mcl-1 proteins after 24 h (Fig. 4B). Furthermore, ectopic expression of cFLIPs significantly blocked DNA fragmentation, which was observed in Caki/vector cells treated with oxaliplatin (Fig. 4C). It has been reported that ectopic expression of c-FLIPL inhibits the TNF-α-induced ROS accumulation [Nakajima et al., 2006]. Therefore, we also defined the possible role of cFLIPs in ROS generation on oxaliplatin-induced apoptosis, we measured the changes in the fluorescence using H_2DCFDA. As shown in

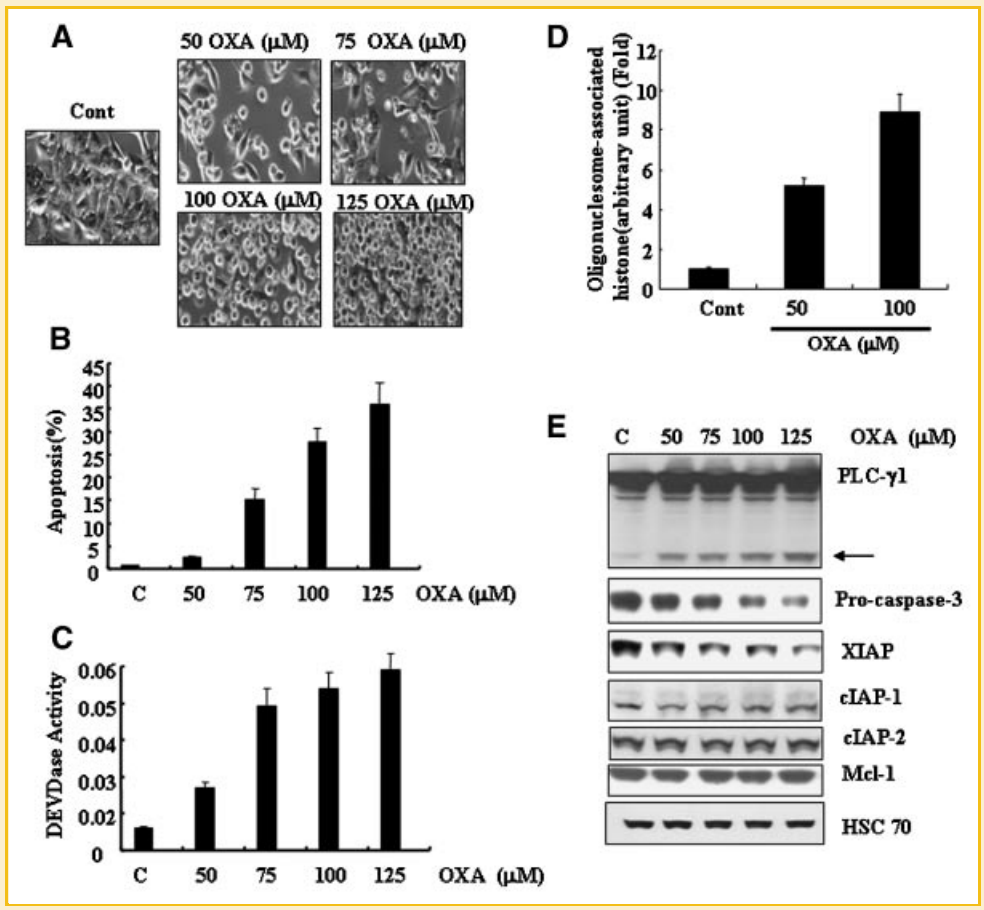

Fig. 1. Oxaliplatin (OXA) induced cell death in a dose-dependent manner. A: Caki cells were treated with various concentrations of oxaliplatin (OXA) for 24 h. The morphologies of cells were determined by interference light microscopy. Magnification, 200×. B,C: Caki cells were treated with various concentrations of oxaliplatin for 24 h, harvested and analyzed by flow cytometry (B) and DEVDase activity(C). D: Oxaliplatin-induced DNA fragmentation in Caki cells. Caki cells were incubated with indicated concentrations of oxaliplatin. DNA fragmentation in Caki cells was determined by the DNA fragmentation detection kit. Data shown are means ± SD (n = 3). E: Caki cells were treated with the indicated concentrations of oxaliplatin for 24 h. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-PLC-γ1, anti-procaspase-3, anti-XIAP, anti-cIAP1, anti-cIAP2, anti-Mcl-1, and anti-HSC70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results.

Fig. 4D, oxaliplatin-mediated increase in fluorescence was markedly inhibited by cFLIPs expression.

SUSTAINED EXPRESSION OF XIAP IS CAUSED BY THE INCREASED XIAP PROTEIN STABILITY IN cFLIPs-OVEREXPRESSING CELLS

To examine whether the decrease of XIAP protein is related to the down-regulation of mRNA expression in Caki cells, we measured XIAP mRNA levels by RT-PCR. As shown in Figure 5A, XIAP mRNA levels remain constant through the oxaliplatin treatment at different doses in Caki/Vector and Caki/cFLIPs cells, suggesting that oxaliplatin-mediated down-regulation of total XIAP protein is regulated by the post-transcriptional levels. Since XIAP has been previously reported to be a substrate of caspases during apoptosis [Deveraux et al., 1999], we tested whether the decrease in the protein levels of XIAP during induced by oxaliplatin was correlated with enhanced caspase activity. As shown in Figure 5B, XIAP down-regulation was not prevented by pretreatment with 50 μM z-VAD-fmk in the presence of oxaliplatin in Caki/Vector and Caki/cFLIPs cells, indicating that the decrease of XIAP protein was mediated by caspase-independent pathways. To further clarify the underlying mechanisms of sustained XIAP protein levels in oxaliplatin-treated cFLIPs overexpressing cells, we analyzed XIAP protein stability. After Caki/Vector and Caki/cFLIPs cells were pretreated with cycloheximide (CHX) for inhibition of new protein synthesis, these cells treated with or without oxaliplatin for different time periods. With increasing duration of single CHX treatment, XIAP protein level dropped to a similar degree in both Caki/Vector and Caki/CFLIPs cells (Fig. 5C, lower panel). On the other hand, XIAP protein was disappeared much more rapidly in Caki/Vector cell than Caki/cFLIPs cells after 18h of oxaliplatin plus CHX treatment (Fig. 5C, upper panel). These results suggest that ectopic expression of cFLIPs does not affect physiological degradation of XIAP, but protects cells from drug-induced degradation.

PI3K/Akt PATHWAY IS IMPORTANT TO INCREASE OF XIAP PROTEIN STABILITY IN cFLIPs OVEREXPRESSING CELLS

It has been known that XIAP is a physiologic substrate of Akt that is stabilized to inhibit apoptosis [Dan et al., 2004]. To determine

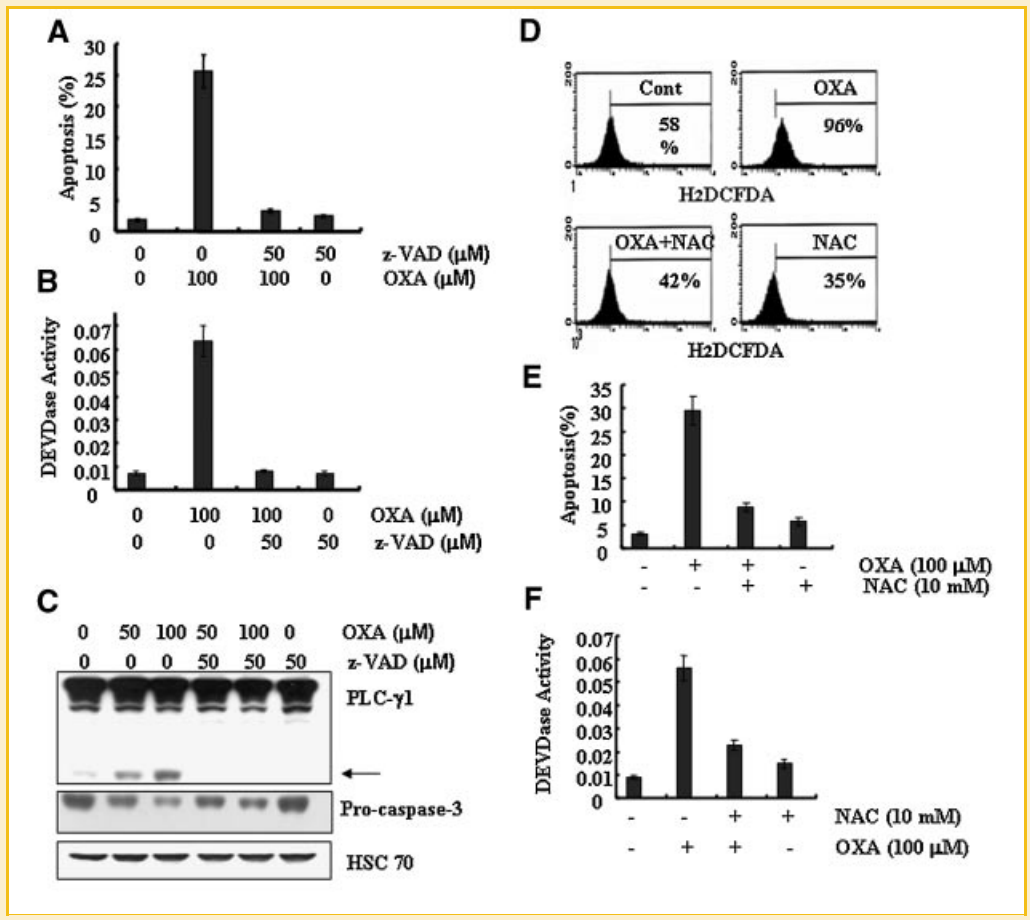

Fig. 2. Oxaliplatin-induced apoptosis appears to be dependent on the generation of reactive oxygen species (ROS) and caspase-dependent pathway. A: Caki cells were incubated with 50 μM z-VAD-fmk or solvent for 1 h before treatment with 100 μM oxaliplatin (OXA) for 24 h. DNA contents of treated cells were evaluated after propidium iodide staining and apoptosis was measured as a sub-G1 fraction by FACS. B: Caki cells were pretreated with 50 μM z-VAD for 1 h before treatment with 100 μM oxaliplatin for 24 h, harvested and analyzed DEVDase activity. C: Caki cells were pretreated with 50 μM z-VAD for 1 h before treatment with 50 or 100 μM oxaliplatin for 24 h, harvested and analyzed Western blotting analysis using anti- PLC-γ1 and anti-procaspase-3 antibodies. Equal loading of the protein samples were confirmed by Western blotting of HSC70. A representative study is shown; two additional experiments yielded similar results. D: Oxaliplatin-induced ROS generation was prevented by pretreatment of NAC. Caki cells were incubated with 5 mM NAC for 1 h before challenge with oxaliplatin for 1 h and fluorescence was measured by flow cytometry. E,F: Caki cells were stimulated with oxaliplatin in the presence or absence of 5 mM NAC (E). After 24 h, cells were harvested and analyzed flow cytometry and DEVDase activity (F).

whether the increased stability of XIAP in oxaliplatin-treated cFLIPs overexpressing cells was related to the levels of phosphorylated Akt, we tested the effect of oxaliplatin on the levels of phospho-Akt in Caki/Vector and Caki/cFLIPs cells. As shown in Figure 6A, the levels of phosphorylated Akt were significantly decreased in response to oxaliplatin in time-dependent manner in Caki/Vector cells. In contrast, the decrease of the levels of phosphorylated Akt was significantly inhibited in Caki/cFLIPs cells. Total Akt protein levels remained constant during treatment with oxaliplatin in both cells. These results suggested that overexpression of cFLIPs appeared to inhibit down-regulation of phosphorylated Akt in oxaliplatin-treated cell. To examine whether ectopic expression of cFLIPs could increase XIAP protein stability via PI3K/Akt signaling pathway, we determined the expression of XIAP and phosphorylation levels of Akt in Caki/cFLIPs cells after treated with or without LY294002, a selective phosphatidylinositol 3-kinase (PI3K) inhibitor, for various times in presence of oxaliplatin plus CHX (vehicle). As shown in Figure 6B, the levels of XIAP protein and phosphorylated Akt were found to be lower in LY294002-treated cells than in vehicle-treated control cells. This result suggests that blockade of PI3K signaling pathways decreased XIAP protein stability in oxaliplatin plus CHX-treated cells. Taken together, ectopic expression of cFLIPs increased XIAP protein stability via PI3K/Akt pathways in Caki cells.

DISCUSSION

In present study, we showed that oxaliplatin treatment induced apoptosis in dose-dependent manner that was mediated by caspase-dependent pathway. Furthermore, we found that overexpression of cFLIPs significantly attenuated oxaliplatin-induced apoptosis in Caki cells by sustained Akt phosphorylation and XIAP protein levels. Furthermore, we have shown that inhibition of Akt phosphorylation by the specific PI3K inhibitor LY294002 significantly

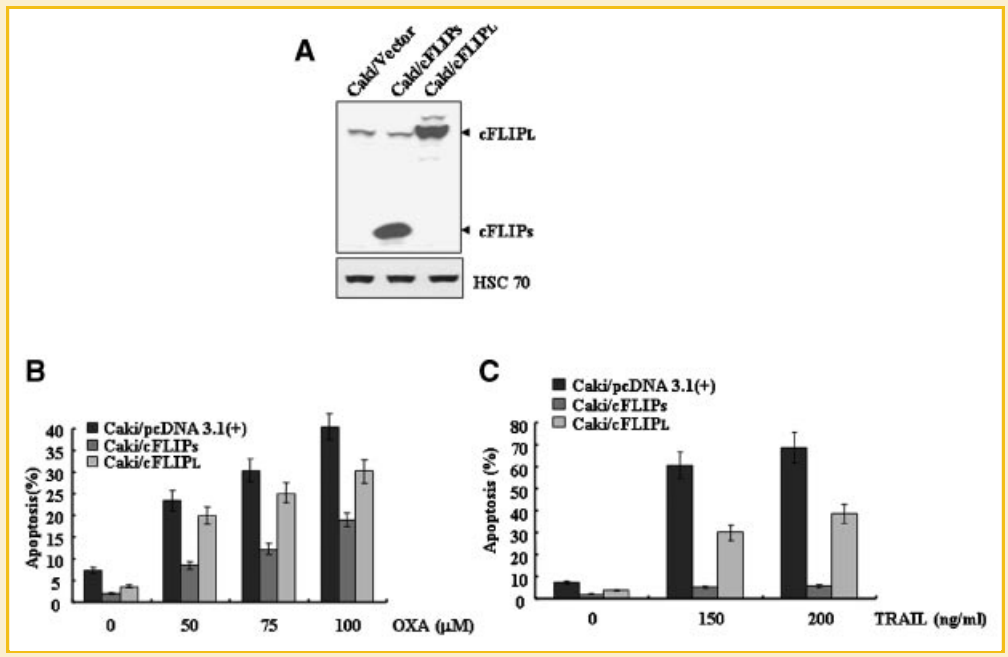

Fig. 3. Establishment of cFLIPs and cFLIP$_L$ overexpression cells. A: Immunoblot analysis of cell lysates (50 μg) from Caki/Vector, cFLIPs, or cFLIP$_L$ transfected cells with anti-cFLIP antibody. Equal loading of the protein samples were confirmed by Western blotting of HSC70. B: cFLIPs overexpression attenuates oxaliplatin-induced apoptosis in Caki cells. Caki/Vector, Caki/cFLIPs, and Caki/cFLIP$_L$ cells were incubated with indicated concentrations of oxaliplatin for 24 h, harvested, and analyzed flow cytometry analysis. C: cFLIPs and cFLIP$_L$ overexpression attenuate TRAIL-induced apoptosis in Caki cells. Caki/Vector, Caki/cFLIPs, and Caki/cFLIP$_L$ cells were incubated with indicated concentrations of TRAIL for 18 h, and analyzed flow cytometry analysis.

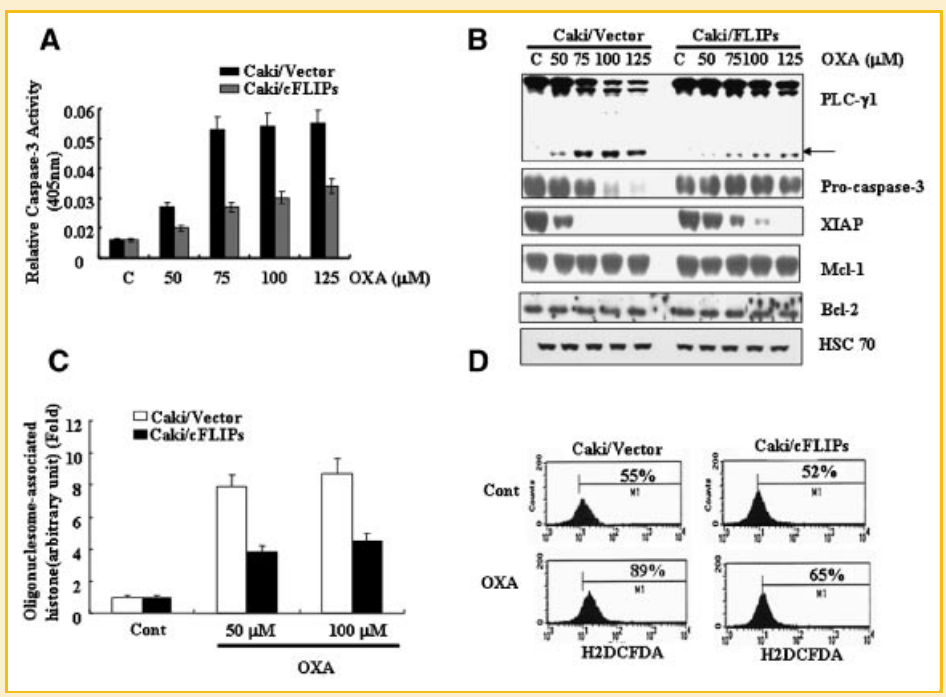

Fig. 4. cFLIPs overexpression attenuates oxaliplatin-induced apoptosis. A: Caki/Vector and Caki/cFLIPs cells were treated for 24 h with the indicated concentrations of oxaliplatin, and measured DEVDase activity. B: Caki/Vector and Caki/cFLIPs cells were treated for 24 h with the indicated concentrations of oxaliplatin, and analyzed Western blotting using anti-PLC-γ1, anti-XIAP, anti-Mcl-1, anti-Bcl-2, and anti-procaspase-3 antibodies. Equal loading of the protein samples were confirmed by Western blotting of HSC70. A representative study is shown; two additional experiments yielded similar results. C: Oxaliplatin-induced DNA fragmentation in Caki/Vector and Caki/cFLIPs. Cells were incubated with the indicated concentrations of oxaliplatin. DNA fragmentation in Caki cells was determined by the DNA fragmentation detection kit. Data shown are means ± SD ($n = 3$). D: Comparison of ROS generation in Caki/Vector and Caki/cFLIPs after oxaliplatin treatment. Caki/Vector and Caki/cFLIPs cells were incubated with oxaliplatin for 18 h and fluorescence was measured by flow cytometry.

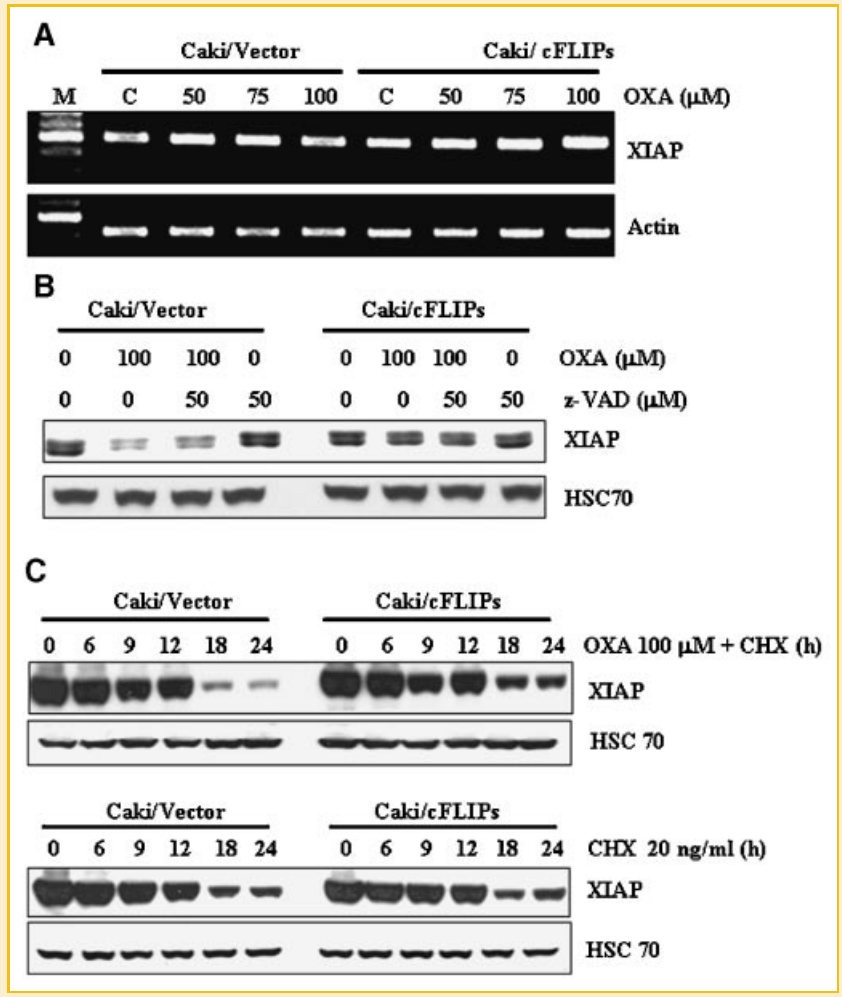

Fig. 5. Sustained expression of XIAP was caused by the increased XIAP protein stability in cFLIPs-overexpressing cells. A: Caki/Vector and Caki/cFLIPs cells were treated with the indicated concentrations of oxaliplatin. Total RNA was isolated and RT-PCR analysis was performed as described in Materials and Methods Section. A representative study is shown; two additional experiments yielded similar results. B: Caki/Vector and Caki/cFLIPs cells were treated with 100 μM oxaliplatin in the presence or absence of z-VAD (50 μM). Western blotting was performed using anti-XIAP and HSC70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results. C: Caki/Vector and Caki/cFLIPs cells were treated with 100 μM oxaliplatin in the presence or absence of 20 ng/ml cycloheximide (CHX) for the indicated times. Western blotting was performed using anti-XIAP and HCS70 antibody to serve as control for the loading of protein level (top). Caki/Vector and Caki/cFLIPs cells were treated with 20 ng/ml CHX for the indicated times. Western blotting was performed using anti-XIAP and HCS70 antibody to serve as control for the loading of protein level (bottom). A representative study is shown; two additional experiments yielded similar results.

enhanced down-regulation of XIAP by decreasing its protein stability in oxaliplatin-treated Caki/cFLIPs cells.

Initially, we investigated the apoptotic potential of oxaliplatin in human renal Caki cells. Oxaliplatin is a platinum-based chemotherapy drug in the same family as cisplatin and carboplatin and forms mainly intra-strand links between two adjacent guanine residues or a guanine and an adenine, disrupting DNA replication and transcription [Fink et al., 1997]. Oxaliplatin has shown activity in several malignancies such as human colon, breast, and renal cancer and it is commonly used to treat patients unresponsive to 5-fluorouracil (5FU) based therapy [Arango et al., 2004; Honecker et al., 2006]. However, the details underlying the cytotoxic effects of oxaliplatin remain poorly understood. We demonstrated that oxaliplatin treatment induced caspase-dependent apoptosis, DNA fragmentation, and cleavage of PLC-γ1 in dose-dependent manners. Recent study has shown that oxaliplatin treatment increased ROS production and its anti-tumor activity was prevented by addition of exogenous GSH [Laurent et al., 2005]. Consistent with previous study, we also found that oxaliplatin treatment modulated the ROS production, which was attenuated by pretreatment with NAC. We also found that overexpression of cFLIPs markedly inhibited ROS production after oxaliplatin treatment. Therefore, ROS generation is critical for oxaliplatin-mediated apoptopsis in cancer cells.

Numerous studies have demonstrated that cFLIP overexpression confers resistance to death receptor-mediated apoptosis as well as anticancer therapeutic drugs-mediated apoptosis [Irmler et al., 1997; Scaffidi et al., 1999; Nam et al., 2003]. In addition, tumors that up-regulate cFLIP expression as a defense against death receptor might simultaneously acquire resistance to anticancer drugs-induced apoptosis [Matta et al., 2002]. It is well-established

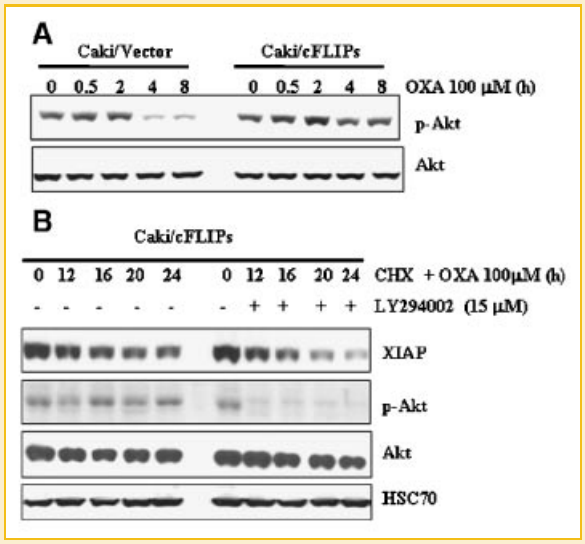

Fig. 6. PI3K/Akt pathway is important to increase of XIAP protein stability in cFLIPs overexpressing cell. A: Caki/Vector and Caki/cFLIPs cells were treated with 100 μM oxaliplatin for the indicated times. Western blotting was performed using anti-phospho-Akt and anti-Akt antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results. B: Caki/cFLIPs cells were treated with 100 μM oxaliplatin in the presence or absence of LY294002 (15 μM) for the indicated times. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-XIAP, anti-phospho-Akt, anti-Akt, and anti-HSC70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results.

molecular mechanism of cFLIP to inhibit death receptor-mediated apoptosis. However, the molecular events and genetic programs responsible for cFLIP-mediated protection against various anti-cancer drugs are poorly understood. $cFLIP_L$ prevents p38 MAPK activation, thereby protecting cells from bile acid-induced apoptosis [Grambihler et al., 2003]. More recently, it has been shown that overexpression of $cFLIP_L$ significantly decreased oxaliplatin-induced apoptosis, whereas cFLIPs overexpression failed to inhibit oxaliplatin-induced apoptosis in human colon HCT116 cancer cell line [Longley et al., 2006]. In contrast to this result, we observed overexpression of cFLIPs attenuated oxaliplatin-induced apoptosis in human renal carcinoma Caki cells. One of the possible reasons for this discrepancy is that intrinsic differences between colon and renal carcinoma cells in cancer cell types.

Chemotherapeutic drugs can trigger apoptosis and consequently down-regulate the anti-apoptotic molecules involved in regulating apoptosis. One such molecule that has been implicated in regulating apoptosis is XIAP protein [Li et al., 2000]. XIAP is a member of intracellular anti-apoptotic proteins (IAPs) that were first identified in baculovirus and a direct inhibitor of caspase-3 and caspase-9 and modulates the Bax/cytochrome *c* pathway by inhibiting caspase-9 [Song et al., 2003]. Cisplatin down-regulates XIAP, leading to a corresponding activation of caspase 3 in cisplatin-sensitive cells. In contrast, no decrease in XIAP has been shown after exposure to an equal molar concentration of cisplatin in cisplatin-resistant cells, which appears to depend on activation of the PI3-kinase/Akt signaling pathway [Asselin et al., 2001; Fraser et al., 2003; Richardson and Kaye, 2005; Yang et al., 2005]. In this study, we found that the protein levels of XIAP were higher in oxaliplatin-treated Caki/cFLIPs overexpressing cell than in oxaliplatin-treated Caki/Vector cell, which was not caused by differences of XIAP mRNA expression patterns. These results suggest that the sustained XIAP protein level was regulated at posttranscriptional levels in oxaliplatin-treated cFLIPs overexpressing cell. It has been reported that XIAP protein was degraded by caspase-dependent pathway or by ubiquitination system response to DNA damage, including treatment with chemotherapeutic agents [Deveraux et al., 1998; Cheng et al., 2002; Lane et al., 2006]. In current study, we demonstrated that oxaliplatin-induced down-regulation of XIAP protein was mediated by caspase-independent pathway in Caki cells and that the sustained expression of XIAP protein in oxaliplatin-treated cFLIPs overexpressing cells was caused by the increase of protein stability. Akt has also been shown to phosphorylate XIAP at Ser87 and stabilize XIAP, and thus inhibiting its degradation [Dan et al., 2004]. Based on this study, we postulated that the increased protein stability of XIAP was related to PI3K/Akt signaling pathway in Caki/cFLIPs cells. To address this hypothesis, we treated Caki/Vector and Caki/cFLIPs cells with oxaliplatin and analyzed phosphorylated Akt level by Western blotting. As shown in Figure 6, the levels of phosphorylated Akt was higher in Caki/cFLIPs than in Caki/Vector cells after oxaliplatin treatment, indicating that overexpression of cFLIPs might be effect on PI3K/Akt signaling pathway. In addition, we found that XIAP down-regulation was significantly potentiated by PI3 kinase inhibitors LY29402 in cFLIPs overexpressing cells. These data indicate that XIAP protein was stabilized by the activation of PI3K/Akt signaling pathways, which might be a mechanism accounting for cFLIPs-induced oxaliplatin resistance in Caki cells.

Although, oxaliplatin directly decreased p-Akt level in Caki/Vector cells, but not in Caki/FLIPs cells, LY294002 could decrease p-Akt level in Caki/cFLIPs cells. This suggests that oxaliplatin and LY294002 might have differential effect on Akt phosphorylation in cFLIPs overexpressing cell via different pathway(s). However, further studies are needed to investigate the role of cFLIPs in regulating of PI3K/Akt signaling pathway as well as its relevance to examine the effect of increased/decreased cFLIPs expression on cell viability and drug sensitivity in vivo.

Collectively, the data presented here demonstrate for the first time that overexpression of cFLIPs protected oxaliplatin-induced apoptosis in renal cancer cells and XIAP is regulated by cFLIPs through PI3K/Akt signaling pathway in oxaliplatin-treated cells.

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